

Botulinum Neurotoxins

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1. Introduction

Botulism is a neuromuscular disease caused by neurotoxin produced from the bacterium *Clostridium botulinum*. The botulinum neurotoxins (BoNTs) are among the most potent known biological toxins and have an estimated human median lethal dose (LD₅₀) in the nanogram/kilogram range. Botulinum toxins have historically been employed as biological weapons (BW) through state-sponsored programs in Japan, Germany, the United States, Russia and Iraq as well as by independent terrorist organizations. The extreme potency of the toxin, its persistence within affected neurons, the need for protracted intensive care, and the lack of an effective post-intoxication therapeutic intervention make BoNTs a potentially deadly offensive biological weapon.

2. Botulinum neurotoxins as biological weapons

2.1 Historical applications of botulinum toxins in biological warfare

There have been reports of botulinum neurotoxin being used as an offensive weapon since the early 1900s. Anecdotal accounts describe the implementation of crude anaerobic fermenters made by burying canteens filled with water, green beans and slivers of meat to facilitate *C. botulinum* production for use against Mexican federal troops in 1910 (Carrus, 2001). Documented state sponsored programs utilizing BoNT as a potential BW were reported as early as the 1930s when the military medical commander of Unit 731, General Shiro Ishii, confessed to feeding food contaminated with lethal cultures of *C. botulinum* to prisoners of war (Williams & Wallace, 1989). In the early 1940s, the US developed a BoNT-based BW program in response to Allied intelligence reports that Germany was attempting to develop the neurotoxin as an offensive weapon to be used against an invasion force (Franz et al., 1997). The initial efforts of the US program were primarily directed at the isolation and purification of the toxin and the elucidation of the mechanism of pathogenicity of BoNT, then referred to as agent "X" (Cochrane, 1947). In response to the potential threat from Germany, over 1 million doses of a botulinum toxoid vaccine were prepared for Allied troops involved in the D-Day invasion in Normandy (Bryden, 1989). The 1972 Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological

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(Biological) and Toxin Weapons and on their Destruction signed by President Richard M. Nixon went into effect in March of 1975, effectively terminating the US BW efforts. All of the biological agent stockpiles created in the US offensive program, including botulinum neurotoxins, were destroyed. Despite being signatories on the convention, the Soviet Union continued not only to pursue its biowarfare program, including research on botulinum neurotoxins, but expanded their program during the post-Soviet era (UNSC, 1995; Bozheyeva et al., 1999). Botulinum toxins were reportedly one of several biological agents tested at the Soviet Aralsk-7 site on Vozrozhdeniye (Renaissance) Island in the Aral Sea. The Soviets were also believed to have attempted to use recombinant DNA technology to introduce BoNT genes into alternative strains of bacteria for the purpose of enhancing toxin production (Alibek & Handelman, 1999). Iraq, which also ratified the 1975 convention, reportedly also significantly expanded its biological weapons program (UNSC, 1995). After the Persian Gulf War, Iraq admitted to a United Nations Special Commission inspection team that approximately 4,900 gallons of concentrated botulinum neurotoxins had been produced for use in specially designed bombs, missiles and tank dispersion instruments in 1989 (Zilinskas, 1997). Iraq maintains that no biological weapons were employed during the Persian Gulf War or Operation Iraqi Freedom and that its stockpiles have since been destroyed (Blix, 2004).

The Japanese cult Aum Shrinkyo (now referred to as Aleph) attempted to develop both chemical and biological weapons after its political aspirations were defeated in the 1990 Japanese Diet elections. Formed in 1987 by Shoko Asahara, this group developed rapidly and was reported to have 50,000 members and over US\$ 1 billion in financial resources by 1995 (Sugishima, 2003). The Aum executed a deadly sarin nerve gas attack in Matsumoto City on June 27, 1994 which killed seven people. On March 20, 1995 they perpetrated a sarin gas assault on a Tokyo subway which killed 12 people and injured over 1000 individuals. Less than a month later they attempted a cyanide gas release in a restroom of the subway's Shinjuku Station. Senior Aum members obtained soil samples in an attempt to isolate toxin-producing strains of *C. botulinum*. Despite the cult having members with a variety of scientific expertise, they are believed to have experienced difficulty cultivating toxigenic strains of *C. botulinum* and were most likely unable to produce any substantial amount of toxin (Sugishima, 2003; Leitenberg, 1999).

2.2 Domestic threat targets

The possibility of dissemination of toxins such as BoNT through municipal water supplies or centralized agricultural or food distribution hubs has been explored in a number of scenarios. There has never been a confirmed case of waterborne botulism. In 1980, Notermans & Havelaar reported on the stability of BoNT/A, /B and /E when introduced into samples of reservoir surface water, drinking water prepared by sand-filtration, and sterile distilled water. Toxicity was reduced 99% in just over 3 days in the surface water, 9 days in filtered water and relatively stable in sterile distilled water. Treatment with FeCl_3 , a common coagulant processing step employed in municipal water treatments to remove iron, removed 75-95% of the toxins. Treatment with 1 mg of ozone per liter for 2 min destroyed $\geq 99\%$ of the toxins and 0.3-0.5 mg/L sodium hypochlorite ablated 99% of the toxicity in 30 seconds. Despite the extreme potency of BoNTs, their application as a bioterrorism weapon by introduction into a water supply in the US would most likely be

unsuccessful when conventional municipal water treatment methods are employed (Notermans & Havelaar, 1980; Wannemacher, et al., 1993). Another consideration is that large-capacity reservoirs undergo a relatively slow turn-over rate and so a comparably large inoculum of botulinum toxin would be needed (Burrows et al., 1997). Given the technical difficulties involved in producing such a large amount of toxin, this route seems unlikely. Small-scale (personal-use) water filtration systems utilizing ceramic or membrane filters with a 0.2 to 0.4 μm pore size were found to be insufficient to effectively remove BoNT/B introduced into drinking water. However, a reverse osmosis device was found to remove BoNT levels below the detection limit of the mouse bioassay (Hörman, et al. 2005).

American agriculture has been described as being "concentrated, highly accessible, vertically integrated" and as such, susceptible to the malicious introduction of biological toxins or human pathogens at processing or distribution points that would reach consumers before a significant threat was realized (Parker, 2002). Wein and Liu published a mathematical model to predict the effects of the deliberate introduction of botulinum toxin at various points through the nine-stage milk collection, processing and distribution network in the US, as it is associated with a single processing facility (Wein and Liu 2005). The study has a number of variables such as the amount of toxin employed, the total volume of the milk, the specific effect of the pasteurization process on the toxin and variable delivery time and consumptions rates, but all scenarios support a significant outbreak of up to 100,000 individuals. The report generated considerable controversy but the editors of the publishing journal defended the release, citing its potential contribution to developing defensive counter-measures as well as informing federal and state governments of the putative threat (Alberts, 2005). The devastating potential of an intentional bioterrorism attack using this avenue was illustrated in 1985 when almost 200,000 people were infected with an antibiotic-resistant strain of *Salmonella* caused by an inadvertent contamination at a single northern Illinois dairy processing plant (Ryan, et al., 1987).

The potential susceptibility of centralized food distribution platforms in the US can be illustrated by outbreaks attributed to improper processing of mass distributed consumable products. In September 2006, a total of 6 individuals from 2 US states and 1 Canadian province were admitted to local hospitals with cranial neuropathies and flaccid paralysis necessitating mechanical ventilation (CDC, 2007). All of the individuals tested positive for BoNT/A and one patient eventually died. The outbreaks were traced to a commercially produced carrot juice manufactured in a single plant which was distributed under three different brand labels. The intoxications were attributed to a lapse in refrigeration during transport or storage and a lack of chemical barriers to *C. botulinum* germination during processing. In the summer of 2007, 8 cases of botulism in 3 US states led to a massive recall of canned meat products. The outbreaks were traced to product from a single production line in a single plant that was packaged and distributed under 90 different labels (NCFPD, 2008) and which necessitated the recall of tens of millions of cans of suspect food. While these outbreaks represent unintentional distribution of BoNT, they do reveal the complexity of the food production and distribution network in the US and how it might be compromised by a BW attack using a potent toxin such as BoNT.

3. Microbiology and BoNT toxicity

3.1 The organism and its toxins

Clostridium spp. are gram-positive, spore-forming, obligate anaerobic bacteria that are ubiquitous in soil and both freshwater and marine sediment (Dunbar, 1990). BoNTs are produced primarily in *C. botulinum* but may also be produced in the closely related *C. butyricum*, *C. baratii* and *C. argentinense* (Hatheway, 1993). *C. botulinum* strains are classified into four groups (denoted I to IV) based on metabolic activity (Hatheway, 1988) and genetic composition (Collins, 1998; Hill et al., 2007). Group I includes type A strains and proteolytic strains of types B and F; Group II includes type E strains and nonproteolytic strains of types B and F; Group III includes nonproteolytic strains of types C and D; and Group IV includes only strains that produce type G. There are seven immunologically distinct serotypes of BoNTs designated by the letters A through G (BoNT/A to BoNT/G) (Smith & Sugiyama, 1988). BoNT/A, based on animal studies, has a lethal human dose (LD₅₀), assuming 70 kg weight, of approximately 0.09 - 0.15 µg by intravenous administration, 0.7 - 0.9 µg by inhalation, and 70 µg by oral administration (Scott & Suzuki, 1988; Arnon et al., 2001). The toxicity for other serotypes is unknown but all have been shown to uniformly fatal in animals studies. Human botulism is caused primarily by BoNT/A, /B and /E (Arnon et al., 2001), and rarely by BoNT/F (Barash et al., 2005; Gupta et al., 2005). BoNT/C and /D primarily cause botulism in animals. BoNT/G, produced by *C. argentinense*, has been associated with sudden death but not neuroparalytic illness in a few patients in Switzerland (Sonnabend et al., 1981). All seven serotypes can cause inhalational botulism in primates (Middlebrook & Franz, 1997). Recent characterization of an increasing number of unique BoNT subtypes has revealed small to significant variations at the amino acid level (Smith, et al., 2005; Hill, et al. 2007; Smith, et al., 2007). These variations impact binding and protection of neutralizing antibodies (Smith, et al., 2005) and raise concerns that they may prove problematic to the development of prophylactic and therapeutic agents developed against a dissimilar subtype.

BoNTs are produced as a part of a protein complex in which the toxin is non-covalently bound to two or more protein components. This includes the well characterized hemagglutinins (HA) and a nontoxin, nonhemagglutinin (NTNH). The complexes can be distinguished on the basis of their size and serotype association and include the M-(300 kDa in types A-F), L-(500 kDa in serotypes A, B, C, D and G) and LL-(900 kDa in serotype A) forms (Collins & East, 1988). Although neither accessory protein has been implicated in the toxin-mediated blockade of neurotransmitter release, they are believed to protect the toxin against the harsh environment of the gastro-intestinal tract (Simpson, 2004). The neurotoxin is initially produced as a 150-kDa single-chain prototoxin that is proteolytically cleaved into an N-terminal 50-kDa light chain (LC) and a C-terminal 100-kDa heavy chain (HC) di-chain that is linked by a single disulfide bond (DasGupta, 1989). The BoNT HC is further delineated into two domains; the N-fragment or translocation domain (Hn) and the C-fragment or receptor-binding domain (Hc). These three domains mediate intoxication of the neuron in a defined tripartite mechanism. The toxin is introduced into the nerve cell by receptor-mediated endocytosis through binding of the Hc domain to specific ectoreceptors on peripheral cholinergic nerve cells (Dong et al., 2006; Rummel et al., 2007). The acidic pH of the endosome initiates a conformation change in the dichain toxin that results in the Hn

forming a protein channel that facilitates the translocation of the LC out of the endosomal lumen and into the cytosol. The LC is a zinc-containing endopeptidase that target SNARE (soluble *N*-ethylmaleamide sensitive factor attachment protein receptors) proteins that form the synaptosomal fusion complex in a serotype dependent fashion. The SNARE protein SNAP-25 (synaptosomal-associated protein of 25 kDa) is cleaved at different sites by BoNT/A, /C, and /E, synaptobrevin (also referred to as VAMP; vesicle-associated membrane protein), is cleaved at different sites by BoNT/B, /D, /F, and /G, and syntaxin is cleaved by BoNT/C (Simpson, 2004). Proteolytic cleavage of SNARE proteins prevents the release of acetylcholine across the synaptic cleft of the neuromuscular junction, resulting in a flaccid muscle paralysis which is the primary clinical sign of botulism.

3.2 Epidemiology

Botulism is typically reported in four clinical categories. *Foodborne botulism* is caused by the ingestion of the pre-formed toxin in contaminated food (CDC, 1998). Most outbreaks are associated with home-canned foods in which inadequate processing results in *C. botulinum* spores germinating, reproducing and producing the toxin (Shapiro, et al., 1998). These conditions include an anaerobic environment with temperatures ranging from 4°C to 40°C, a pH range from 4.6 to 7.0, and water activity greater than 0.94 (a_w is intensity with which water associates with various non-aqueous constituents and solids) (Baird-Parker & Freame, 1967; Stringer, et al., 2005). Despite increased educational awareness of the non-permissive conditions for food preparation, foodborne botulism remains a persistent threat. There were, on average, approximately 24 reported cases per year from 1900-2000, but there have been a smaller number of cases (average of 18 year) reported from 2001-2009 (CDC-NBS). In the period from 1950-1996, there were 444 outbreaks in which one or more cases of botulism from a contaminated food source was implicated. Of these outbreaks, 37.6% were caused by type A, 13.7% by type B, 15.1% by type E and 0.7% by type F while 32.9% of the incidents were caused by unidentified serotype(s) (CDC, 1998). Improvements in both differential diagnostic methods and the technology utilized in serotyping have resulted in a decrease in unidentified serotypes. In the United States from 1990-2000 BoNT/A was responsible for 50% of all cases of botulism, where types B and E were responsible for 10% and 37% of the intoxications, respectively; only 3.6% of the cases were from an unidentified strain (CDC, 1998).

Infant botulism is a toxicoinfection caused by inhalation or ingestion of clostridial spores that can colonize and produce toxin in the intestinal tract of infants less than 12 months of age (Shapiro, 1998). The ability of the bacteria to thrive and elicit toxin is thought to be attributed in part to a deficiency in protective gastrointestinal bacterial flora and the relatively low levels of inhibitory bile-aid found in children under 12 months (CDC, 1998). This form of the disease was officially recognized in a 1976 report in which two infants presenting with acute infantile hypotonia and weakness were diagnosed with botulism (Pickett, et al., 1976). This has been the most commonly reported form of the disease in the United States since 1980, with an average of approximately 80-100 cases confirmed annually (Shapiro, 1998). In the US between 1976 and 1996, there were 1442 individual cases of infant botulism reported to the CDC (CDC, 1998). Of these, 46.5% were caused by type A, 51.9% by type B. Interestingly, the incidence of infant botulism in the US has a geographical component with 47.6% of the reported cases occurring in California with Delaware, Hawaii,

and Utah also experiencing high incidence (CDC, 1988). The correlation between the increased incidences of infant botulism outbreaks associated with certain geographical locations has not been elucidated.

Wound botulism (WB) results from the growth of *C. botulinum* spores in a contaminated wound with *in vivo* toxin production. This form of the disease was first reported in 1951 as a relatively rare illness associated with post-operative complications (Davis et al., 1951). Historically, approximately 75% of the wound botulism cases in the US have been reported in California (Weber, et al., 1993) and this represents over 90% of the reported incidences in the world (Benson, 2001). There were 127 cases of wound botulism in California from 1951 to 1998. Of these, 105 were attributed to intravenous drug users and all but one were admitted black-tar heroin users (Benson, 2001). The increased incidence of WB in California has not abated and between 1993 and 2006 an additional 17 cases were identified in intravenous drug users; 16 of which were diagnosed with one or more recurrent episodes (Yuan et al., 2011).

Adult intestinal toxemia botulism is a rare toxicoinfection that occurs in older children and adults with abnormal gastrointestinal tract physiology, such as colitis or intestinal surgical procedures (Freeman et al., 1986; Fenecia et al., 1999). The disease has also been correlated with alteration of protective endogenous microflora by broad-spectrum antibiotics after inflammatory intestinal disease or surgery (Chia et al., 1986).

In addition to the four naturally occurring forms of the disease described previously, there are two additional forms of *inadvertent botulism* that result from the application of the purified toxin. *Iatrogenic botulism* results from the injection of BoNT for either cosmetic or therapeutic purposes. Two adult patients developed symptoms of botulism when given therapeutic doses of BoNT/A drawn from two different production lots (Bakheit, 1997). These cases have since been attributed to either patient sensitivity to the drug or the inadvertent injection of the toxin directly into the vascular capillaries. An adolescent being treated for spastic quadripareisis with Myobloc (botulinum toxin serotype B) developed clinical signs of a systemic BoNT injection (Partikian, 2007). While there are no clear dosing guidelines for BoNT formulations for therapeutic interventions in children, the clinical diagnosis of botulism was again believed to have been the result of inadvertent injection into a blood vessel or diffusion from nearby muscle sites. In November of 2007, four adults were given cosmetic injections of undiluted BoNT/A intended for laboratory research (Chertow et al., 2006). Serial dilutions of two of the individual's serum samples indicated they were given approximately 21 to 43 estimated human lethal doses. All four patients survived but only after prolonged hospitalization with anti-toxin treatment and ventilator support ranging from 40 to 104 days.

Inhalational botulism is intoxication by an inhalational exposure of the aerosolized toxin. The only reported human inhalation incidence occurred in Germany in 1962 during a necropsy when three laboratory workers were exposed to animals subjected to aerosolization of a highly purified, lyophilized BoNT/A (Holzer, 1962). The patients were hospitalized five days postexposure, administered equine antitoxin, and discharged after 9 days. While not a naturally occurring form of the disease, inhalational botulism has implications as a potential weapon of bioterrorism (Middlebrook & Franz 1997; Zilinskas, 1997). An aerosol dispersion of BoNT could create a toxic gas cloud that could encompass a large area and is considered to be a likely scenario for a terrorism attack.

3.3 Clinical symptoms

The clinical presentation of botulism is characterized by distinctive neurological symptoms of the voluntary motor and autonomic cholinergic-associated junctions in the infant, wound and intestinal forms of the disease (CDC, 1998). Foodborne botulism is often accompanied by acute gastrointestinal distress including nausea, abdominal cramps, vomiting and diarrhea that precede the neurological symptoms (Hughes et al, 1981), particularly in types B and E. The initial symptoms of food botulism can manifest anywhere from within a few hours to several days postintoxication. The time to onset of symptoms, the severity and the duration of the disease is largely dictated by the exposure dose and the serotype (Arnon, 2001; Woodruff et al., 1992). Infant botulism may present with constipation, poor feeding, diminished sucking, neck and peripheral weakness, weak crying increased drooling (Corblath, et al., 1993) and ventilatory failure (Arnon, 1992; Long et al., 1985). Wound botulism does not display the gastrointestinal symptoms observed in the foodborne form. Fever, if present, is generally attributed to a wound infection rather than botulism.

The neuroparalytic effects of botulism present as an acute, afebrile, descending, bilateral flaccid paralysis. The initial neurological symptoms generally involve cranial nerves III, IV and VI (Sobel, 2005) and include ocular disorders such as blurred vision, diplopia, ptosis and photophobia (Terranova et al., 1979). This is generally followed by dysfunction of cranial nerves VII and IX which cause, dysphagia, dysphonia and dysarthria. The neurological impairment may then spread to the upper extremities, the trunk and then the lower extremities. Respiratory distress can be caused by a weakened glottis that tends to obstruct the airway during attempted inspiration or from paralytic weakness of the diaphragm and parasternal and intercostal muscles. Fatigue, sore throat, dry mouth, constipation and dizziness have also been reported to be associated with botulinum intoxications (Hughes, 1981). Fatalities are most often the result of respiratory failure or secondary infections typically associated with prolonged mechanical ventilation. Bleck summarized the clinical findings of several published reports of foodborne botulism by symptom and serotype as shown in Table 1 (Bleck, 2000).

The primary neurological disorders associated with botulism are common to the foodborne, intestinal, and wound forms of the disease (Sobel, 2005). The duration and severity of the neuroparalytic effects of the disease can be influenced by both the amount and serotype of the toxin introduced into the system. Insight into the impact of the different serotypes in humans has only recently been investigated and much of the data comes from studies involving the therapeutic applications of BoNTs. Of the three most prevalent serotypes involved in human incidences of botulism, BoNT/A has been shown to have the most persistent action and can last 12-16 weeks when used in therapeutic applications (Eleopra, 2004). Serotype B has also been used in therapeutic applications but only exhibits the comparable efficacy to BoNT/A when used in higher doses (Sloop, et al., 1997; Settler, 2001). Electrophysiological studies conducted in juvenile monkeys using purified BoNT/A (BOTOX, Allergan, Irvine, CA) and BoNT/B (Neurobloc, Elan, Shannon, Ireland) indicated BoNT/A diffusion was more pronounced (Arezzo, 2001). The few studies pertaining to serotype C in humans (Eleopra et al., 1997; Eleopra et al., 1998a, Eleopra et al., 1998b) indicate that it is similar to BoNT/A in terms of the toxicity and duration of activity. A 2004 study evaluated the electrophysiological responses to human volunteers injected with low doses

	Type A (%)	Type B (%)	Type E (%)
<i>Neurological symptoms</i>			
Dysphagia	96	97	82
Dry mouth	83	100	93
Diplopia	90	92	39
Dysarthria	100	69	50
Upper extremity weakness	86	64	NA
Lower extremity weakness	76	64	NA
Blurred vision	100	42	91
Dyspnea	91	34	88
Paresthesiae	20	12	NA
<i>Gastrointestinal Symptoms</i>			
Constipation	73	73	52
Nausea	73	57	84
Vomiting	70	50	96
Abdominal cramps	33	46	NA
Diarrhea	35	8	39
<i>Miscellaneous symptoms</i>			
Fatigue	92	69	84
Sore throat	75	39	38
Dizziness	86	30	63
<i>Neurological Findings</i>			
Ptosis	96	55	46
Diminished gag reflex	81	54	NA
Ophthalmoparesis	87	46	NA
Facial paresis	84	48	NA
Tongue weakness	21	31	66
Pupils fixed or dilated	33	56	75
Nystagmus	44	4	NA
Upper extremity weakness	91	62	NA
Lower extremity weakness	82	59	NA
Ataxia	24	13	NA
DTRs diminished or absent	54	29	NA
DTRs hyperactive	12	0	NA
<i>Initial mental status</i>			
Alert	88	93	27
Lethargic	4	4	73
Obtunded	8	4	0

DTRs, deep tendon reflexes; NA, not available.

Table 1. Summary of Symptoms of Patients with Botulism Caused by Serotypes A, B and E. from Springer Scientific Publishing.

of BoNT/A, /B, /C or /F (Eleopra et al., 2004). The results were consistent with other research efforts in that BoNT/B, used in higher doses, and BoNT/C have a similar profile as BoNT/A. As reported in previous studies (Mezaki et al., 1995; Chen et al., 1998), BoNT/F was found to have a shorter duration compared to BoNT/A.

3.4 Diagnosis of botulism

A rapid and accurate identification of botulism is not difficult when the disease is strongly suspected, such as found in the setting of a large outbreak. But because cases of naturally occurring botulism most often occur singularly, the individual diagnosis may prove more challenging. Botulism is thought to be substantially underdiagnosed (CDC, 1988) and with low-level exposure, minor neurological manifestations of the disease may resolve without medical intervention. A differential diagnosis of botulism without concurrent knowledge of a confirmed outbreak can be difficult and other paralytic illnesses may need to be excluded. These include Guillain-Barre syndrome, myasthenia gravis, tick paralysis, and Eaton-Lambert syndrome (Dembek, et al., 2007). Less likely conditions such as tetrodotoxin and shellfish poisoning, aminoglycoside toxicity and a variety of other neurotoxic products and neurological abnormalities may initially present with similar symptoms. However, a thorough medical examination of the patient and their medical history can generally exclude any competing diagnosis. A patient presenting with an acute, bilateral, descending flaccid paralysis that is afebrile and has normal sensorium should suggest a clinical case of botulism.

The most reliable method for the detection of BoNTs and for diagnosing botulism is the mouse bioassay. This test can be performed by the Centers for Disease Control and Prevention (CDC) or state public health laboratories. The assay involves injecting mice with samples collected from patients displaying symptoms of botulism. Mice will typically begin showing signs of botulism within 8 h. The serotype of samples can also be ascertained in this manner by neutralizing the toxin with serotype-specific antibodies prior to injecting the mice (Shapiro et al., 1998). In cases of foodborne or infant botulism, stool samples can also be cultured to look for *C. botulinum*. Samples of the suspected food should also be cultured anaerobically with heat or alcohol treatment to select for spores.

4. Medical countermeasures

4.1 Current medical intervention

Standard therapy for botulism involves administration of botulinum antitoxin in an attempt to prevent neurologic progression of a moderately progressive illness, or to reduce the duration of respiratory failure in individuals with a severe, rapidly progressive illness. This is done in conjunction with careful monitoring of respiratory vital capacity and aggressive ventilatory care for individuals that display respiratory failure. The only specific pharmacological treatment for botulism is administration of equine-derived botulinum antitoxin. On March 12, 2010, a new heptavalent botulinum antitoxin (HBAT, Cangene Corp.) became available through a US CDC-sponsored FDA Investigational New Drug (IND) protocol for the treatment of naturally acquired non-infant botulism (CDC, 2010). This antitoxin replaced the previously FDA-approved equine bivalent botulinum antitoxin AB and an investigational monovalent equine antitoxin E (BAT-AB and BAT-E,

Sanofi Pasteur). HBAT contains equine-derived antibody against all BoNT serotypes: 7,500 U anti-A; 5,500 U anti-B; 5,000 U anti-C; 1,000 U anti-D; 8,500 U anti-E; 5,000 U anti-F; and 1,000 U anti-G per vial. This antitoxin is composed of <2% intact immunoglobulin G (IgG) and ≥90% Fab and F(ab')2 immunoglobulin fragments created by despeciation (CDC, 2010). The recommended adult dosing is one 20 mL vial of HBAT (McLaughlin and Funk, 2010). BabyBIG®, human botulism immune globulin intravenous (BIG-IV), is an FDA-approved drug for the treatment of infant botulism types A and B. Available through the California Infant Botulism Treatment and Prevention Program, BabyBIG® is obtained from the pooled plasma of adults vaccinated with the pentavalent (A-E) botulinum toxoid who displayed high titers of neutralizing antibodies against BoNT/A and /B. Because BabyBIG® is of human origin, it does not carry the risk for anaphylaxis inherent with equine products, nor does BabyBIG® demonstrate a risk for possible lifelong hypersensitivity to equine antigens. BabyBIG® has been shown to significantly shorten the hospitalization period and reduce treatment costs up to \$75,000.00 per incident (Thompson et al., 2005; Fox, 2005).

Antitoxin can neutralize toxin molecules that are not yet bound to nerve endings and may limit the progression of the disease and prevent further nerve damage by clearing them from circulation. Thus, the antitoxin should be administered immediately upon a definitive diagnosis of botulism, preferably within 24 h after the onset of symptoms (Tacket et al., 1984; Chang and Ganguly, 2003). Compared to standard care alone, immediate administration of antitoxin has been shown to shorten time on respiratory support, and also reduced the hospitalization period (Shapiro et al., 1997; Tacket et al., 1984). It is not clear how long the toxin can persist in the bloodstream before clearance. Ravichandran reported that BoNT/A had a serum half-life of approximately 4 hours in small animal studies (Ravichandran et al., 2005). This study also suggested that blood does not sequester or modify the toxin in any detectable way. Thus, the blood may act as a reservoir for the toxin until it either enters the target cells or is eliminated from the body. Detectable levels of toxin were observed in one of the four patients in the Florida outbreak 8 days after receiving a massive overdose of an unlicensed preparation of BoNT/A during a cosmetic procedure (Chertow et al., 2006). Under such circumstances, antitoxin administration, even if delayed, may still be effective in limiting the duration of the illness. The use of equine-based antitoxins, but not the human product, has been associated with symptoms of hypersensitivity (including urticaria, serum sickness, and anaphylaxis). Hence, dermal testing is required before antitoxin administration. Due to the risk of adverse reactions, prophylactic antitoxin is not recommended in patients who are exposed to BoNT but have no symptoms. These patients may undergo gastric lavage or induced vomiting in an attempt to eliminate the toxin before absorption (Chan-Tack & Bartlett, 2010).

4.2 Prophylaxis

In the United States, there is currently no FDA-licensed prophylactic product against botulism. Some of the earliest vaccine efforts were initiated during the second World War due to concerns that the toxin might be employed as an offensive biological weapon against the allied forces by Germany. A bivalent A/B toxoid vaccine was produced from 3-day culture autolysates of *C. botulinum* that were chemically neutralized with formaldehyde, filtered and adsorbed onto an alum adjuvant. While the vaccine did elicit the production of

neutralizing antibodies, it also displayed a number of localized and systemic reactogenic effects (Reames et al., 1947). An improved product in which the A and B toxins were purified to 10-15% homogeneity by acid-precipitation of the culture supernatants and adjuvanted onto aluminum phosphate was found to be well-tolerated in humans, displayed only minor localized reactogenic effects and produced significantly improved antibody titers over the previous bivalent toxoid (Flock et al., 1961). A pentavalent ABCDE toxoid vaccine with 1% thymersol as a preservative prepared by Parke, Davis and Company was administered to approximately 400 individuals (Flock et al., 1963). The vaccine displayed minor reactogenic effects and elicited detectable antibody titers to all five serotypes. This product was administered to over 1600 individuals from 1970 to 1981 under an Investigational New Drug (IND) application. The Michigan Department of Public Health produced a pentavalent botulism (ABCDE) toxoid (PBT) using similar procedures as the Parke Davis product, but which contained roughly 50% of the formaldehyde and only 0.01% thymersol, that has been administered under an IND to at-risk laboratory workers and military personnel. However, the declining immunogenicity, dwindling supplies and local reactogenic effects of the PBT (Rusnak & Smith, 2009; CDC, 2009) have led to recent efforts to create new vaccines.

More contemporary toxoid vaccines have been created by chemical neutralization of the purified toxin (Keller, 2008; Jones, et al., 2008). However, large scale production of these products would require a secure, CDC-licensed facility to both propagate large amounts the bacterium and manipulate the purified toxin. Subsequent efforts have largely relied on the expression of recombinant protein antigens encoding one or more of the BoNT domains.

Codon optimized genes encoding the BoNT (Hc) antigens produced in a *P. pastoris* expression platform have been successfully developed as a recombinant subunit vaccine against serotypes A-F and have been demonstrated to elicit protective immunity in both rodent (Smith, 2009; Rusnak & Smith 2009) and non-human primates (Boles et al., 2006; Morefield, 2008). In February of 2011, the Dynport Vaccine Company announced that phase II clinical trials of a recombinant bivalent Hc vaccine against serotypes A and B (rBV A/B) had been completed and that full licensure would be sought. (CSC, 2011). Recombinant, catalytically inactive BoNT holoproteins made by mutagenesis of key amino acids residues have also been employed as potential vaccine candidates. A recombinant BoNT/C protein with active site mutations H²²⁹G, E²³⁰T and H²²³N produced in *E. coli* displayed no catalytic activity and elicited protective immunity against the parental toxin when delivered either subcutaneously or orally (Kiyatkin et al., 1997). Pier described the expression of a recombinant BoNT/A gene bearing R³⁶³A and Y³⁶⁵F mutations in the LNT01 nontoxicogenic strain of *C. botulinum* (Pier et al., 2008). The recombinant protein was unable to cleave SNAP-25 and elicited protective immunity in mice when challenged with BoNT/A. Recombinant BoNT/A with active site mutations H²²³A, E²²⁴A and H²²⁷A produced in *P. pastoris* was found to be completely non-toxic and provided protective immunity in mice against 1000 MLD₅₀ of not only the parental toxin, but against subtypes /A2 and /A3 as well (Webb et al., 2009).

4.3 Post-intoxication interventions

Once the neurotoxic LC is endocytosed within the cytosol of peripheral cholinergic neurons, circulatory antibodies will no longer be effective at neutralizing the catalytic

activity and ablating the toxicity. A number of recent research efforts have focused on the development of small-molecule inhibitors to reduce or eliminate the cleavage of SNARE proteins in neurons. The drawbacks associated with the use of peptides as drug candidates (e.g., poor tissue penetration, serum resistance, oral bioavailability, and quick elimination), and the potential usefulness of small molecules as pre- and post-exposure therapeutic agents have led many laboratories to instead pursue small-molecule approaches. To date, research has focused predominantly on developing small-molecule inhibitors that target the BoNT/A LC protease, due to the serotype's persistence and highly toxic nature. A number of BoNT/A small-molecule inhibitors, identified using conventional and novel approaches, have been reported that exhibit varying degrees of inhibitory capacity (Burnett et al., 2003; 2009; Boldt et al., 2006; Park et al., 2006; Tang et al., 2007; Capkova et al., 2007; 2009; Moe et al., 2009; Roxas-Duncan et al., 2009; Burnett et al., 2010). Most of these studies were conducted *in vitro* using truncated forms of the LC which might not be structurally representative of the intracellular form of BoNT. The inhibitors reported by Roxas-Duncan et al (2009), identified via a hierarchical screening strategy, were evaluated *in vitro* using both forms of LC (truncated and full-length), and *ex vivo* using mouse phrenic nerve hemidiaphragm preparations. Despite intensive efforts on small-molecule inhibitor discovery and development, no compound has been identified that would be suitable for preclinical testing. At present, there are only two reports of BoNT/A small-molecule inhibitors that have been tested *in vivo*. Janda and co-workers described a mouse toxicity bioassay in which two different inhibitors were injected intravenously immediately after an injection of 5-10 i.p. LD₅₀ of BoNT/A. One compound, given at a 2.5 mM dose, showed a 36% increase in time to death, while the other, given at a 1 mM dose, resulted in a 16% overall survival rate (Eubanks et al., 2007). In another study, a single dose of three different inhibitors was administered i.p. at 2 mg/kg 30 min prior to a challenge of 5 MLD₅₀ of BoNT/A (Pang et al., 2010). The control mice died within 12 hrs. All 3 inhibitors provided 100% protection at 12 hrs and one compound provided 70% and 60% survival rates at 24 and 48 hrs; all three inhibitors provided a 10% overall survival rate with no signs of botulism at 5 days.

Recently, other regions of the LC, in addition to the BoNT active site, generated attention as potential targets for inhibition. Merrino et al., (2006) has focused on a family of bis-imidazole BoNT/A inhibitors targeting the peripheral sites of substrate binding. Silhar et al., (2010) reported on D-chicoric acid, a natural product isolated from Echinacea, that inhibits BoNT/A LC by binding to an exosite.

4.4 Human recombinant monoclonal antibodies

Currently, immunotherapy is deemed as the most effective immediate response to BoNT exposure. However, BabyBig® is exclusively approved for use in infants, and equine antisera can induce serum sickness and anaphylaxis (Arnon et al., 2001; Arnon, 2004). Monoclonal antibody (mAb) combinations (oligoclonal antibodies) may be viable substitutes for polyclonal antisera (Nowakowski et al., 2002; Razai et al., 2005). Construction of scFv phage Ab libraries has enabled the generation of large panels of high-affinity binding monoclonal antibodies. Mouse neutralization studies revealed that effective protection is observed only when combinations of three or more mAbs are used (Nowakowski et al., 2002; Marks, 2004). Under the aegis of the National Institute of Allergy and Infectious

Diseases (NIAID), antibody combinations that effectively protect against multiple BoNT/A, /B, and /E subtypes are currently being produced and tested to support FDA licensure. Antibodies that protect against the four remaining toxin serotypes (BoNT/C, /D, /F, and /G) are also in development.

4.5 Antibodies specific for the catalytic light chain

While a majority of the BoNT immunotherapy research has been focused on antibodies that bind the HC, efforts were also directed to explore the potential for antibodies that bind the enzymatic LC. Using a novel hybridoma method for cloning human antibodies (Adekar et al., 2008; Dessain et al., 2004), a fully human antibody specific for the BoNT/A LC was isolated which potently inhibited BoNT/A in vitro and in vivo, via mechanisms not previously associated with BoNT-neutralizing antibodies (Adekar et al., 2008). In another study, Dong et al. (2010) created a library of non-immune llama single-domain VHH (camelid heavy-chain variable regions) displayed on the surface of the yeast *Saccharomyces cerevisiae*. Library selections against BoNT/A LC yielded 15 yeast-displayed VHHs, eight of which inhibited the cleavage of substrate SNAP-25 by BoNT/A LC. The most potent VHH (Aa1) had a solution K_d of 1.47×10^{-10} M and an IC_{50} of 4.7×10^{-10} M. X-ray crystal structure of the BoNT/A LC-Aa1 VHH complex revealed that the Aa1 VHH binds the alpha-exosite region of BoNT/A LC. Recently, Tremblay et al. (2010) reported on the selection of small (14 kDa) binding domains specific for the protease of BoNT serotypes A or B from libraries of VHHs or nanobodies cloned from vaccinated alpacas. Several VHHs were demonstrated to exhibit high affinity (K_d near 1 nm) and were potent inhibitors of BoNT/A LC (K_i near 1 nM); a VHH inhibitor of BoNT/A LC was able to protect BoNT/A-mediated SNAP25 cleavage.

4.6 Inhibitors of internalization and translocation

Several compounds that inhibit the acidification process of endosomes by various mechanisms have been evaluated for both toxicity and ability to inhibit BoNT-induced synaptic failure. Lysosomotropic agents ammonium chloride and methylamine hydrochloride have been shown to antagonize the toxin internalization step by delaying the time-to-block of nerve-evoked muscle contractions after exposure to BoNT/A, /B, /C1, and TeNT (Simpson, 1983). However, these amines act by inhibiting the acidification process of endosomes; they do not selectively inactivate the toxins nor irreversibly modify tissue function at concentrations that inhibit the onset of BoNT-induced paralysis. Other candidates that have been examined were uncouplers of oxidative phosphorylation CCCP, FCCP (Adler et al., 1994), and vesicle H⁺-ATPase inhibitors including Bafilomycin A, which was shown to antagonize BoNTs A-G (Simpson et al., 1994). Some of these compounds were toxic or had low safety margins, hence they were deemed unsuitable as therapeutic candidates.

Anti-malarial compounds chloroquine and hydroxychloroquine have also been evaluated for potentially inhibiting BoNT-mediated internalization (Simpson, 1982). The efficacy of these agents was found similar to that of ammonium chloride and methylamine hydrochloride; both groups also exhibited a comparable therapeutic window. Deshpande and co-workers (1997) extended the studies on antimalarial agents by examining a large

group of 4- and 8-aminoquinolines. Unfortunately, these compounds failed to extend the therapeutic window. The most effective compounds were 4-aminoquinolines and quinacrine that delayed BoNT/A-induced neuromuscular block by more than threefold compared to the control (toxin only) values. Maximum protection was solely achieved when the tissues were exposed to the compounds before or at the same time as the toxin treatment; a delay of >20 min abolished the inhibitory capacity of these compounds.

An additional approach to prevent or reduce BoNT internalization has been attempted by treating nerve-muscle preparations with the protein ionophores nigericin and monensin (Adler et al., 1994; Sheridan, 1996). These ionophores block vesicle acidification by acting as H⁺ shunts to neutralize pH gradients, thereby interfering with the delivery of active LC in the cytosol. Though the efficacy of these ionophores was observed to be comparable to other inhibitors of internalization, they were more toxic; high concentrations resulted in a depression of neuromuscular transmission (Adler et al., 1994; Sheridan, 1996).

4.7 Compounds that restore neuronal function

A known potassium channel blocker, 3,4-diaminopyridine (3,4-DAP) was evaluated for its ability to antagonize BoNT-induced depression of tension in rat diaphragm muscles (Adler et al., 1995). BoNT-induced paralysis was nearly completely inhibited after addition of 100 uM of 3,4-DAP, and this effect was sustained even after 4 h of treatment. The antagonistic effects of 3,4-DAP was also demonstrated *in vivo*, provided its concentration in the plasma is maintained at ~30 μM during the course of intoxication (Adler et al., 2000). However, 3,4-DAP is generally toxic, thus, its high drug concentration requirement prohibits routine therapeutic use (Millard, 2006).

Mastoparan, a phospholipase activator, was evaluated for its ability to attenuate BoNT intoxication. Addition of mastoparan and 80 mM K⁺ completely prevented BoNT inhibition of radiolabeled acetylcholine in PC12 cells, but this effect was blocked by either EGTA or the N-type calcium channel blocker ω -conotoxin (Ray et al., 1999). These findings imply that the effects of mastoparan are dependent on Ca²⁺ influx via the neuronal type voltage-sensitive Ca²⁺ channels.

4.8 Drug delivery vehicle research

One significant challenge in the development of BoNT small-molecule therapeutics is the delivery of the compounds to the cytosol of peripheral cholinergic nerve cells (PCNC), which are the sites of BoNT action. In combination with the discovery and development of BoNT small-molecule inhibitors, cell-specific intracellular targeting is critical to increase the therapeutic index and minimize potential systemic toxicity associated with the drug treatment. Several studies have examined the potential of using specific recombinant BoNT domains or a neutralized full-length BoNT holoprotein as drug delivery system. Goodnough et al. observed that BoNT/A and unlabeled rHC were able to compete for binding, implicating specific neuronal targeting (Goodnough et al., 2002). The efficiency of neuron-specific cargo delivery into the cytosol was evaluated by coupling labeled dextran to a recombinant BoNT/A HC using a 3-(2-pyridylthio)-propionyl hydrazide linker (Zhang et al., 2009). A fluorescent tracking dye was conjugated to the dextran moiety and incubated in mouse cultured mouse spinal cord neurons. The binding of the fluorescent tag and its

internalization into the endosomes were observed, but minimal levels were detected in the cytosol. The movement of a small, membrane-permeable dye from the endosome into the cytosol is hypothesized to be due to passive diffusion instead of an active translocation event. Ho et al (2010) reported on a recombinant BoNT/A HC with an amino terminal GFP fusion that was internalized into mouse neurons; however, the GFP cargo was observed to be almost exclusively limited to endocytotic vesicles, with little detectable translocation into the cytosol.

Although the of BoNT heavy chain comprises the domains necessary for binding and internalization into endosomes, recent studies suggest that all or part of the LC is essential to translocate a ligated cargo into the cytosol.

Recombinant BoNT/D fusion proteins bearing amino terminal GFP, luciferase, dihydrofolate reductase or BoNT/A LC protein were found to promote translocation of cargo proteins into the cytosol in an enzymatically active form (Bade et al., 2004). A recombinant neutralized BoNT/A bearing an E²²⁴A E²⁶²A double mutation, labeled with Alexa-488, has been shown to specifically bind and internalize into human SH-SY5Y neuroblastoma cells (Sing et al., 2010). Additionally, this protein was found to marginally bind to the surface of human rhabdomyosarcoma cells with a toxicity limit of 1 µg in mice. No additional information is available regarding the drug conjugation study on this protein. Moreover, recombinant versions of full-length BoNT/A holotoxin devoid of catalytic activity were recently developed (Pier et al., 2008; Webb et al., 2009; Yang et al., 2008) which could be potentially used to deliver conjugated therapeutic cargoes.

5. Emergency preparedness and public response

The 2007 anthrax attacks in the United States illustrated the need to develop a comprehensive preparedness plan for identifying and managing healthcare resources in the event of a biological attack. Anthrax, plague, botulinum toxins, smallpox, tularemia, and viral hemorrhagic fever viruses have been identified as having a significant potential for use in a bioterrorism attack because they can be easily disseminated or transmitted, have high morbidity or mortality rates and would cause widespread social disruption (Rotz et al., 2002). A bioterrorism attack involving dissemination of BoNT by existing food or water distribution networks is theoretically possible but this route is associated with significant logistical difficulties (Franz, et al., 1997; Zilinskas, 1999) and most experts believe that an aerosol dispersion poses the greatest threat (Arnon, 2002). Because botulism is a relatively rare disease, clinicians and the healthcare infrastructure in general have limited experience in diagnosis of any form of the disease. A 2005 study reported that of 631 physicians participating in a mock bioterrorism event, slightly less than half provided an accurate diagnosis and plan of management for botulism (Cosgrove, 2005). In the event of a biological attack with BoNT, a rapid and accurate diagnosis of the agent by physicians and clinical laboratories would be crucial to an equally rapid response and mobilization of equipment and biological agents for management. Health care providers who suspect botulism should immediately call their state health department's emergency 24-hour emergency telephone number. The state health department will contact the CDC to report suspected botulism cases, arrange for a clinical consultation by telephone and, if indicated, request release of botulinum antitoxin. State health departments should call the CDC 24-hour telephone number at 770-488-7100. The call will be taken by the CDC Emergency

Operations Center, which will page the Foodborne and Diarrheal Diseases Branch medical officer on call. The CDC established the Laboratory Response Network (LRN) in 1999; a national network of about 150 federal, state, local and military labs that can respond to biological and chemical terrorism, and other public health emergencies (CDC-EPR). The LRN hierarchy also includes *reference labs* that can perform tests to detect and confirm the presence of a threat agent to ensure a rapid local response in the event of a bioterrorism incident without having to rely on confirmation from CDC labs. *Sentinel labs* are comprised of thousands of hospital-based labs that have direct contact with patients. These labs might well be the first facility to spot a suspicious specimen and refer it to the appropriate reference lab (Sentinel, ASM).

The ability of the government and healthcare infrastructure to respond to a bioterrorism attack using BoNT in a densely populated urban environment would largely be dictated by the ventilator and antitoxin supplies and how quickly they could be mobilized to the outbreak area. Approximately 120,000 of the 200,000 doses of the HBAT contracted by the Biomedical Advanced Research and Development Authority (BARDA) have been delivered for use in the US national strategic stockpile and the remaining 80,000 doses are scheduled for delivery by 2014. A 2010 study estimated that there are approximately 63,066 full-featured mechanical ventilators in the US, 46.4% of which were capable of ventilating pediatric and neonatal patients (Robinson et al, 2010). Additionally, there was an estimated 82,775 additional positive pressure ventilators (PPV) that could be brought on-line in response to an acute respiratory failure (ARF) surge event. This study may represent a conservative estimate as it only accounted for survey responders and it did not consider the various units rented by hospitals, those facilities who failed to respond to the survey or those at nursing facilities or schools.

6. Conclusion

BoNT has been investigated as a BW by a number of different state-sponsored initiatives and terrorist organizations and is considered a biological threat to both our military and the public. Effective medical countermeasures against BoNT intoxication are limited. Currently, the only available treatment other than supportive care is a new investigational botulinum heptavalent equine-based antitoxin. However, antitoxin cannot intervene in the pathogenesis of the disease once the toxin enters the nerve cell, and cannot support all those infected in the event of a biological terrorist attack. Hence, there is a critical need for postintoxication therapy that can be administered rapidly and effectively to a large infected population. Because small molecules provide an opportunity to treat botulism both before and after cellular intoxication has occurred, considerable research efforts have been devoted to the development of these types of inhibitors. A number of strong contributions to the field have been made, yet no small molecule inhibitor was identified that possesses the appropriate characteristics (safety, efficacy, solubility) required to be a pharmaceutical intervention.

Research studies addressing these obstacles are underway. Continuing efforts will be facilitated particularly by the availability of structural information and by knowledge of the mechanism of the BoNT LC-mediated proteolysis of SNARE proteins. Additionally, potential novel strategies to therapeutic development, e.g., host-directed therapeutics, and

targeting pathways involved in the cellular response to BoNT intoxication, are being explored. An understanding of these mechanisms may provide insight into the design and development of innovative and effective therapeutic strategies to counteract BoNT intoxications.

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